09/937057 .082600

JC11 Rec'd PCT/PTO SEP 1 9 2001

FORM PTO-1390 (REV. 11-2000) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: 260/264

U S APPLICATION NO (If known, see 37 CFR 1 5): 09/937057 Not Yet Assigned

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR00/00676	17 March 2000	19 March 1999

TITLE OF INVENTION: USE OF STABILISED OLIGONUCLEOTIDES FOR PREPARING A MEDICAMENT WITH ANTITUMOR ACTIVITY

APPLICANT(S) FOR DO/EO/US: Antoine Carpentier

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [XX] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [XX] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [XX] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [XX] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [X] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [XX] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [X] Other items or information:

Copy of Sequence Listing on Diskette;
International Preliminary Examination Report (in French),
Avis Informant Le Deposant De La Communication De La Demande Internationale Aux Offices Designes;
Notification Relative A La Presentation Ou A La Transmission Du Document De Priorite;
Return Postcard

CERTIFICATE OF MAILING (37 C.F.R. §1.10)

I hereby certify that this paper (along with any referred to as being attached or enclosed is being deposited with the United States Postal Service on the date shown below with sufficient postage as 'Express Mail Post Office To Addressee' in an envelope addressed to the Commissioner for Patents, Washington, D.C. 20231.

Express Mail Label No. EL723999553US Lynne Fulmer

September 19, 2001 Lynne Fulmer
Date of Deposit Signature of Person Mailing Paper

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
Not Yet Assigned 09/937057	PCT/FR00/00676	260/264

	CALCULATIONS PTO USE ONLY	
21. ☐ The following fees are submitted:		
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	31 - 20 =	11	x **$18.00**	$ 198.00	
Independent claims	3 - 3 =		x **$80.00**	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$	
TOTAL OF ABOVE CALCULATIONS =				$ $1,058.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.			+	$	
SUBTOTAL =				$	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$	
				Amount to be refunded:	$
				charged:	$1,058.00

a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 12-2475 in the amount of $ 1,058.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☐ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. _____. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

Kurt T. Mulville
Lyon & Lyon LLP
633 West Fifth St., Ste. 4700
Los Angeles, CA 90071
949/567-2300 X 1124 Telephone
213/955-0440 Facsimile

Dated: September 19, 2001

SIGNATURE

NAME Kurt T. Mulville
Reg. No. 37,194

REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:
ANTOINE CARPENTIER

Serial No.: Not Yet Assigned
(Based on PCT/FR00/00676, Priority Filing Date March 19, 1999)

Filed: Herewith

For: : USE OF STABILISED OLIGONUCLEOTIDES FOR PREPARING A MEDICAMENT WITH ANTITUMOR ACTIVITY

PRELIMINARY AMENDMENT

BOX PCT
Commissioner for Patents
Washington, D.C. 20231

Sir:

IN THE CLAIMS:

Prior to calculation of the fee, please cancel claims 1-17 and add new claims 18-48 as follows:

18. A composition comprising immunostimulatory oligonucleotide comprised of an octomeric CG motif of the sequence AACGTTAT in a pharmaceutically acceptable medicament with anti-tumor activity.

OC-92316 1OF MAILING
(37 C.F.R §1 10)

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Express Mail Label No.

September 19, 2001
Date of Deposit

Lynne Fulmer
Name of Person Mailing Paper

Lynne Fulmer
Signature of Person Mailing Paper

19. The composition of claim 18, wherein the immunostimulatory oligonucleotide is single-stranded or double -stranded.

20. The composition of claim 18, wherein the immunostimulatory oligonucleotide is stabilised.

21. The composition of claim 20, wherein the immunostimulatory oligonucleotide is stabilised by a modified backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphodiester-phosphorothioate mixture, and a stabilisation at a 3' or 5' end.

22. The composition of claim 18, wherein the immunostimulatory oligonucleotide is combined with an encapsulating agent, colloidal dispersion system, or a polymer.

23. The composition of claim 18, wherein at least one cytosine of the immunostimulatory oligonucleotide is a modified cytosine.

24. The composition of claim 23, wherein the modified cytosine is 5-bromocytosine.

25. The composition of claim 18, wherein the immunostimulatory oligonucleotide is combined with immune system cells, adjuvants of immunity, cytokines, antitumor antibodies, or tumor extracts.

26. The composition of claim 18, wherein the immunostimulatory oligonucleotide is combined with tumor cells, irradiated tumor cells, or genetically modified tumor cells.

27. The composition of claim 18, wherein the immunostimulatory oligonucleotide is between 20 and 100 nucleotides in length.

28. A composition comprised of an immunostimulatory oligonucleotide comprised of:
at least two identical octomeric CG motifs of the sequence:
AACGTTAT in a pharmaceutically acceptable medicament with antitumor activity.

29. The composition of claim 28, wherein the immunostimulatory oligonucleotide is single-stranded or double -stranded.

30. The composition of claim 28, wherein the immunostimulatory oligonucleotide is stabilised.

31. The composition of claim 30, wherein the immunostimulatory oligonucleotide is stabilised by a modified backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, and a phosphodiester-phosphorothioate mixture and a stabilisation at a 3' or 5' end.

32. The composition of claim 28, wherein the immunostimulatory oligonucleotide is combined with an encapsulating agent, colloidal dispersion system, or a polymer.

33. The composition of claim 28, wherein at least one cytosine of the immunostimulatory oligonucleotide is a modified cytosine.

34. The composition of claim 33, wherein the modified cytosine is 5-bromocytosine.

35. The composition of claim 28, wherein the immunostimulatory oligonucleotide is combined with immune system cells, adjuvants of immunity, cytokines, antitumor antibodies, tumor extracts, or tumor antigens.

36. The composition of claim 28, wherein the immunostimulatory oligonucleotide is combined with tumor cells, irradiated tumor cells, or genetically modified tumor cells.

37. An oligonucleotide comprised of:

at least three octomeric CG motifs according to a sequence

(pur-pur-C-G-pyr-pyr- X_1 X_2)

wherein the sequence (pur-pur-C-G-pyr-pyr) X_1 X_2 is selected from the group consisting of AA, AT, CT, and TT.

38. The composition of claim 37, wherein the immunostimulatory oligonucleotide is single-stranded or double -stranded.

39. The composition of claim 37, wherein the immunostimulatory oligonucleotide is stabilised.

40. The composition of claim 37, wherein the immunostimulatory oligonucleotide is stabilised by a modified backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, and a phosphodiester-phosphorothioate mixture and a stabilisation at a 3' or 5' end.

41. The composition of claim 37, wherein the immunostimulatory oligonucleotide is combined with an encapsulating agent, colloidal dispersion system, or a polymer.

42. The composition of claim 37, wherein at least one cytosine of the immunostimulatory oligonucleotide is a modified cytosine.

43. The composition of claim 42, wherein the modified cytosine is 5-bromocytosine.

44. The composition of claim 37, wherein the immunostimulatory oligonucleotide is combined with immune system cells, adjuvants of immunity, cytokines, antitumor antibodies, or tumor extracts.

45. The composition of claim 37, wherein the immunostimulatory oligonucleotide is combined with tumor cells, irradiated tumor cells, or genetically modified tumor cells.

46. The composition of claim 37, wherein two of the three octomeric CG motifs are identical.

47. The composition of claim 37, wherein all of the three octomeric CG motifs are identical.

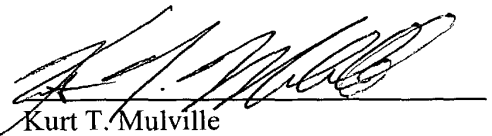
48. The oligonucleotide of claim 37, wherein the sequence of the immunostimulatory oligonucleotide is:

TA AACGTTATAACGTTAT GACGTCAT.

Respectfully submitted,

LYON & LYON LLP

Dated: September 19, 2001

By: 
Kurt T. Mulville
Reg. No. 37,194
Attorneys for Applicants

633 West Fifth Street, Suite 4700
Los Angeles, California 90071-2066
949/567-2300 X 1124
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5000

Patent
260/264

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:

ANTOINE CARPENTIER

Serial No.: 09/937,057

Filed: September 19, 2001
(Priority Date: March 19, 1999)

For: : USE OF STABILISED OLIGONUCLEOTIDES FOR PREPARING A MEDICAMENT WITH ANTITUMOR ACTIVITY

SECOND PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

Sir:

IN THE ABSTRACT:

The abstract was inadvertently omitted from the application. The abstract should be inserted in the above-referenced application and read as follows:

The invention concerns the use of stabilised oligonucleotides comprising at least an octamer motif of the type: 5'-purine-purine-CG-pyrimidine-pyrimidine-X_1X_2-3' wherein the pair X_1 -X_2-is AT, AA, CT or TT, for preparing a medicine with antitumor activity.

OC-92493.1

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October 4, 2001
Date of Deposit

Lynne Fulmer
Name of Person Mailing Paper

Lynne Fulmer
Signature of Person Mailing Paper

IN THE CLAIMS:

Please amend claims 35 and 37 as follows:

35. The composition of claim 28, wherein the immunostimulatory oligonucleotide is combined with immune system cells, adjuvants of immunity, cytokines, antitumor antibodies, or tumor extracts.

37. An oligonucleotide comprised of:

at least three octomeric CG motifs according to a sequence

(pur-pur-C-G-pyr-pyr- X_1 X_2)

wherein the sequence (pur-pur-C-G-pyr-pyr) is palindromic and X_1 X_2 is selected from the group consisting of AA, AT, CT, and TT.

Respectfully submitted,

LYON & LYON LLP

Dated: October 4, 2001

By: _____
Kurt T. Mulville
Reg. No. 37,194
Attorneys for Applicants

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Los Angeles, California 90071-2066
949/567-2300 X 1124
213/955-0440 Facsimile

VERSION WITH MARKINGS TO SHOW CHANGES MADE

35. The composition of claim 28, wherein the immunostimulatory oligonucleotide is combined with immune system cells, adjuvants of immunity, cytokines, antitumor antibodies, or tumor extracts[, or tumor antigens].

37. An oligonucleotide comprised of:

at least three octomeric CG motifs according to a sequence

(pur-pur-C-G-pyr-pyr- X_1 X_2)

wherein the sequence (pur-pur-C-G-pyr-pyr) is palindromic and X_1 X_2 is selected from the group consisting of AA, AT, CT, and TT.

USE OF STABILIZED OLIGONUCLEOTIDES FOR PREPARING A MEDICAMENT WITH ANTITUMOR ACTIVITY

The present invention relates to the use of stabilized oligonucleotides for preparing a medicament with antitumor activity.

The effective treatment of cancers remains one of the major challenges of medicine today.

The effectiveness of conventional surgical therapies or therapies aimed at cytolysis (chemotherapy and radiotherapy) remains very limited in many cancers.

For astrocytomas for example, the treatment of which is based mainly on surgical exeresis and local cerebral irradiation, the survival median is only 4 to 6 months after surgical exeresis and 8 to 10 months with the combination of surgery and radiotherapy. Supplementary chemotherapy prolongs survival in patients under the age of 60, but very modestly, by about 3 months. Under this triple treatment, the survival median remains less than two years for histological grade III (anaplastic astrocytoma) and less than 1 year for grade IV (glioblastoma). The mortality for these two groups is 100% (Daumas-Duport C. *et al.* (1988), Cancer **62**(10) pp 2152-65).

Stimulation of the immune system in the treatment of cancers is a long-standing idea, and very many products have been tested, such as for example bacterial extracts (Jaeckle K. A. *et al.* (1990), *J. Clin. Oncol.* **8**(8) pp 1408-18) or bacterial DNA, in particular that of *Mycobacterium bovis* (MY-1) (Tokunaga T. *et al.* (1984), *JNCI* **72** pp 955-62). MY-1 is, however, ineffective in increasing survival in a model of glioma in mice (Nakaichi M. *et al.* (1995), *J. Vet. Med. Sci.* **57**(3) pp 583-5). IL2 (Herrlinger U. *et al.* (1996), *J. Neurooncol.* **27**(3) pp 193-203) and, more recently, IL12 (Kishima H. *et al.* (1998), *Br. J. Cancer* **78**(4) pp 446-53; Jean W. C. *et al.* (1998), *Neurosurgery* **42**(4) pp 850-6) have also been studied.

Unfortunately, most of these products have a limited effectiveness or unacceptable toxicity and, to date, only the *Mycobacterium bovis* BCG has resulted in clinical applications, but only in very limited indications for bladder cancers (Soloway M. S. *et al.* (1988), *Urol. Clin. North Am.* **15** pp 661-9).

Oligonucleotides are polymers formed by the combination of purine or pyrimidine bases and of sugars, in particular ribonucleotides or deoxyribonucleotides. In the natural form, the linkages are phosphoester linkages which are sensitive to the nucleases of the human body. Thus, oligonucleotides have a very short half-life (of about one minute) when they are injected into humans, which limits their biological effects. Thus, several studies sought to stabilize oligonucleotides by modifying their chemical structure in order to make them resistant to nucleases. Several types of stabilized oligonucleotide have thus been created, such as, inter alia, phosphorothioates or methylphosphonates (Crooke R. M. (1991), *Anti-Cancer Drug Design* **6** pp 609-46). The most commonly used are phosphorothioate oligonucleotides.

Some oligodeoxynucleotides, and in particular some synthetic oligodeoxynucleotides, sometimes have biological effects per se, outside their conventional antisense properties.

Thus, some oligodeoxynucleotides, independently of any known antisense sequence, stimulate, *in vitro* and *in vivo*, the proliferation of B lymphocytes and the activity of NK cells, and induce the secretion by the cells of α-IFN, β-IFN, γ-IFN, IL6, IL12 or TNF-α (Yamamoto S. *et al.* (1992), *J. Immunol.* **148**(12) pp 4072-6; Yamamoto T. *et al.* (1994), *Microbiol. Immunol.* **38**(10), pp 831-6; Yi A. K. *et al.* (1996), *J. Immunol.* **157**(12) pp 5394-402; Ballas Z. K. *et al.* (1996), *J. Immunol.* **157**(5) pp 1840-5; Cowdery J. S. *et al.* (1996), *J. Immunol.* **156**(12) pp 4570-5; Stacey K. J. *et al.* (1996), *J. Immunol.* **157**(5) pp 2116-22). This set of

cytokines direct toward a Th1-type immune response (Chu R.S. *et al.* (1997), *J. Exp. Med.* **186**(10) pp 1623-31).

Authors have shown that the immunostimulatory properties of these oligodeoxynucleotides are in large part dependent on nonmethylated CG motifs (nonmethylated CpG dinucleotides) which are under-represented in mammalian DNA (Kuramoto E. *et al.* (1992), *Jpn. J. Cancer Res.*, **83** pp 1128-31).

While the authors agree on the fact that the nonmethylated CG sequence is essential and that the two nucleotides adjacent to the CG motif are also crucial for the immunostimulatory activity, the data published on the nature of the adjacent sequences are contradictory.

Specifically, Krieg A. M. *et al.* ((1996), *Antisense Nucleic Acid Drug Dev.* **6**(2) pp 133-9) claim a hexameric motif of the type 5' purine purine CG pyrimidine pyrimidine 3', whereas application EP 468 520 claims a palindromic hexameric motif. International application WO 9855495 shows that not all the hexamers as defined by Krieg *et al.* 1996 (mentioned above) are immunostimulatory, and that octamers, of sequence 5'-purine purine CG pyrimidine pyrimidine CC-3' or of sequence 5'-purine purine CG pyrimidine pyrimidine CG-3' should rather be defined, in order to have immunostimulatory activity.

Other immunostimulatory oligodeoxynucleotides, which are not defined as oligonucleotides having a nonmethylated CG motif, have been described in application EP 855 184; they comprise the binding sequence for eukaryotic transcription factors such as NFκB or the AP-1 family. Among these oligonucleotides, some comprise the nonmethylated CG motif, however.

The use of the immunostimulatory properties of oligodeoxynucleotides comprising a nonmethylated CG-type motif is the subject of research in very varied domains:

(1) in the domain of vaccination, they are used, in combination with the antigen, as an adjuvant for stimulating specifically a Th1-type immune response (Davis H. L. *et al.* (1998), *The Journal of Immunology* **160**(2) pp 870-6; application EP 855 184; international application WO 98/18810 in the name of The University of Iowa Research Foundation, of which A. M. Krieg is one of the inventors; international application WO 98/55495),

(2) in the domain of allergy, they are used alone for modulating the immune response (international applications WO 98/18810 and WO 98/55495) and

(3) in the domain of cancer, they are used:

- either in combination with a tumor antigen, as an adjuvant of an antitumor vaccine (application EP 855 184; Weiner G. J. *et al.* (1996), *Proc. Natl. Acad. Sci.* **94**, pp 10833-7; Wooldridge J. E. *et al.* (1997) *Blood* **89**(8) pp 2994-8),

- or alone as antitumor agents (Connell *et al.* (1999), *Proceedings of the American association for Cancer Research* **40** pp 299; application EP 468 520; Carpentier A. F. *et al.* (1999), *Cancer Research* **59**, pp 5429-5432.

In the latter case, the antitumor activity of only a few sequences, among those described, has been effectively demonstrated:

- Weiner G. J. *et al.* and Wooldridge J.E. *et al.* (already cited) who use an oligonucleotide comprising a nonmethylated CG motif of sequence 5'-TCTCCCAG**CG**TG**CG**CCAT-3', show that this oligonucleotide has no antitumor effect when it is used alone;

- Carpentier *et al.*, Tokunaga *et al.* and Connell *et al.* (mentioned above), who use a phosphorothiate oligonucleotide of the octameric type (5'TGACTGTG<u>AA**CG**TTCG</u>AGATGA3'), a nonstabilized palindromic hexameric oligonucleotide (5' ACCGAT <u>GA**CG**TC</u>GCCGGTGACGGCACCACGACGACGGCCACGTGCT 3') and a hexameric phosphorothioate oligonucleotide of the type

5' purine purine CG pyrimidine pyrimidine 3', respectively, show that said oligonucleotides have antitumor activity.

It emerges from the prior art that, other than the nonmethylated CG motif, the exact nature of the active sequences of these immunostimulatory oligodeoxynucleotides, for producing antitumor activity, is not clearly defined; in particular, the data published on the nature of the sequences adjacent to the nonmethylated CG motif (2 bases in 5' and 2 bases in 3' (hexameric motifs) or 4 bases in 3' (octameric motif)) are contradictory.

Recent studies reported by Hartmann G. *et al.* ((2000), *The Journal of Immunology* 164 pp 1617-24) give explanations concerning the difficulty in defining the sequence of such oligonucleotides.

Specifically, the authors show that, according to the nature of their sequence, the immunostimulatory oligodeoxynucleotides have differential effects on NK activation, the proliferation of B lymphocytes and the secretion of IL12, of IL6 and of γ-INF.

These data indicate that not all immunostimulatory oligodeoxynucleotides are equivalent and effective for all the uses as defined above since it is necessary to stimulate different compartments of the immune system in order to obtain the desired activity: adjuvant, antiallergic or antitumor activity.

In addition, the immune mechanisms of tumor rejection are poorly understood and the data for stimulation of the compartments of the immune system, *in vitro*, as defined above do not make it possible to predict in advance the antitumor effectiveness of a given oligonucleotide, and it is therefore important to test their antitumor activity *in vivo*.

Furthermore, the toxicity of oligodeoxynucleotides containing a CG-type motif has been reported when they are used systemically (IV and IP) and is also to

be taken into account for therapeutic applications (application EP 855 184).

Consequently, the immunostimulatory oligonucleotides comprising a CG motif of the prior art (palindromic hexameric motif, motif 5' purine purine CG pyrimidine pyrimidine 3' or octameric motif), which have varying and random antitumor activities and are toxic, do not make it possible to define a set of effective nontoxic immunostimulatory sequences for antitumor use.

Now, the inventors have shown, surprisingly, that certain pairs of bases in 3' of the motif 5' purine purine CG pyrimidine pyrimidine 3' participate, in an essential way, in optimum antitumor activity.

Consequently, the inventors gave themselves the aim of providing a set of immunostimulatory oligonucleotide sequences which better satisfy practical needs in that they:

- have optimum antitumor activity,
- are not toxic and
- are suitable for antitumor use in humans or animals.

Thus, the subject of the present invention is the use of stabilized oligonucleotides which comprise at least one octameric motif of the type 5'-purine-purine-CG-pyrimidine-pyrimidine-X_1X_2-3', in which the pair X_1X_2 is AT, AA, CT or TT, for preparing a medicament with antitumor activity.

For the purpose of the present invention, the term "oligonucleotide" is intended to mean an oligodeoxynucleotide.

According to a preferred embodiment of the invention, the stabilized oligonucleotides comprise at least one octameric motif selected from the group consisting of: AACGTT-X_1X_2, GACGTT-X_1X_2, AGCGTT-X_1X_2, GGCGTT-X_1X_2, AACGTC-X_1X_2, GACGTC-X_1X_2, AGCGTC-X_1X_2 and GGCGTC-X_1X_2 in which X_1X_2 is AT, AA, CT or TT.

According to an advantageous arrangement of this preferred embodiment of the invention, the stabilized oligonucleotides preferably comprise at least one of the following octameric motifs: AACGTT-X_1X_2 and GACGTC-X_1X_2.

In another preferred embodiment of the invention, one at least of the bases of the octameric motif described above can be modified, in particular one at least of the cytosines can be replaced with a 5-bromocytosine.

In another preferred embodiment of the invention, the stabilized oligonucleotide is selected from the group consisting of the sequences SEQ ID NO: 8 to 48.

In accordance with the invention, the stabilized oligonucleotides are selected in particular from the group consisting of phosphorothioate oligonucleotides, phosphorodithioate oligonucleotides, phosphodiester-phosphorothioate mixed oligonucleotides, methylphosphonate oligonucleotides and the oligonucleotides of which at least one end has been stabilized (Crooke R. M. (1991), *AntiCancer Drug Design*, 6 pp 609-46). Preferably, the stabilized oligonucleotides used according to the present invention are phosphorothioates.

In accordance with the invention, the stabilized oligonucleotides can be used in single-stranded or double-stranded form.

Preferably, the stabilized oligonucleotides can be any length longer than 8 bases or 8 base pairs, preferably more than 20 bases or more than 20 base pairs. Preferably, said oligonucleotides comprise between 20 and 100 nucleotides.

In accordance with the present invention, the oligonucleotides can comprise several octameric motifs as defined above, which may or may not be adjacent; they can also comprise other biologically active sequences, such as antisense sequences. The octameric

sequences can themselves be included in antisense sequences.

A subject of the present invention is also the use of the stabilized oligonucleotides as defined above, for preparing medicaments intended for the treatment of cancers in humans, whatever their nature and their degree of anaplasia, in particular cancers of the central and peripheral nervous systems, especially astrocytomas, glioblastomas, medulloblastomas, neuroblastomas, melanomas and carcinomas.

The stabilized oligonucleotides can advantageously be coupled, via covalent, ionic or weak attachments, to a molecule capable of increasing tumor affinity, such as for example an antibody specific for the tumor tissue.

The stabilized oligonucleotides are preferably used via the intratumoral route, but they can also be administered via any other routes, optionally via multiple routes, in particular via the intravenous, intraperitoneal, topical, transdermal, subcutaneous, intra-arterial, pulmonary, nasopharyngeal or oral routes, in solution, in aqueous or oily suspension, as a powder or in any pharmaceutically acceptable form.

They can be administered in one or more doses, or in continuous release, in particular by means of osmotic micropumps, or combined with any physical or chemical means, especially with encapsulating agents such as colloidal dispersion systems and polymers, in order to have a therapeutically effective dose at the tumor site.

Effective doses will be determined as a function of the age, the state of health and the weight of the patient, and of the type of cancer to be treated. Typically, effective doses in humans are such that, in the case of an intratumoral injection, an oligonucleotide dose of 10 to 1000 µg/g of tumor is obtained at least in a part of the tumor.

In accordance with the invention, the use of the oligonucleotides can be combined with other therapies, in particular surgery, radiotherapy, chemotherapy, immunotherapy and differentiating therapies.

Also in accordance with the invention, said oligonucleotides are combined with cells of the immune system, such as macrophages, lymphocytes or antigen-presenting cells, adjuvants of immunity, cytokines, antitumor antibodies, tumor extracts, tumor antigens, or irradiated, genetically modified, or normal, tumor cells.

Besides the arrangements above, the invention also comprises other arrangements, which will emerge from the following description, which refers to the examples of implementation of the use, which is the subject of the present invention, and to the appended drawings in which:

- Figure 1 illustrates the results obtained after an intratumoral injection of the phosphorothioate oligodeoxynucleotide PT1 (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3'), in the glioma model CNS1 in the brain of Lewis rats (Kruse C. A. *et al.* (1994), *J. Neurooncol.* **22** pp 191-200), on the survival time of the control animals (-); PT1 50 µg injected at D1 (-.-.-.), PT1 50 µg injected at D5 (....) and PT1 50 µg injected at D9 (---), after the injection of the tumor cells. The statistical analysis of the results is carried out using the Kaplan-Meier test.

- Figure 2 illustrates the effect of an intratumoral injection on D1 of the phosphorothioate oligodeoxynucleotide PT1 (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3'), at various doses, in the glioma model CNS1 of Lewis rats, on the survival time of the control animals (-); PT1 50 µg (----), PT1 10 µg (-.-.-.) and PT1 1 µg (....).

- Figure 3 illustrates the effect of an intratumoral injection of the phosphorothioate

oligodeoxynucleotide PT1 (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3') or of the phosphorothioate oligodeoxynucleotide IMM (SEQ ID NO: 1 5'-TGACTGTGAAGGTTAGAGATGA-3'), in a subcutaneous glial tumor model. On D2 after injection of the tumor cells, the animals receive, subcutaneously, at the tumor site, sodium chloride (control -◆-), 50 µg of PT1 (-Δ-), 100 µg of PT1 (-•-) or 50 µg of IMM (-□-). The volume of the tumor is evaluated every two days. The results are expressed as mean ± s.e.m. (Anova Test).

- Figure 4 illustrates the effect of an intratumoral injection of the phosphorothioate oligodeoxynucleotide PT1 or of the phosphodiester oligodeoxynucleotide PE1, both having the SEQ ID NO: 2 (5'-TGACTGTGAACGTTCGAGATGA-3'), in a subcutaneous glial tumor model. On D2 after injection of the tumor cells, the animals receive, subcutaneously, at the tumor site, sodium chloride (control -◆-), 100 µg of PE1 (-□-) or 100 µg of PT1 (-Δ-). The volume of the tumor is evaluated every two days. The results are expressed as mean ± s.e.m. (Anova Test).

- Figure 5 illustrates the effect of an intratumoral injection of the phosphorothioate oligodeoxynucleotide PT1 (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3') or of the phosphorothioate oligodeoxynucleotide IMM (SEQ ID NO: 1 5'-TGACTGTGAAGGTTAGAGATGA-3'), in the neuroblastoma model neuro2a in A/J mice (Sigal R. K. *et al.* (1991), *J. Pediatr. Surg.* **26** pp 389-96). On D2 after injection of these tumor cells, the animals receive, subcutaneously, at the tumor site, sodium chloride (control -◆-), 50 µg of PT1 (-■-), 100 µg of PT1 (-π-) or 50 µg of IMM(-□-). The volume of the tumor is evaluated every four days. The results are expressed as mean ± s.e.m. (Anova Test).

- Figure 6 illustrates the effect of a subcutaneous or intraperitoneal injection of the phosphorothioate oligodeoxynucleotide PT1 (SEQ ID NO: 2

5'-TGACTGTGAACGTTCGAGATGA-3'), at the dose of 50 µg, in the neuroblastoma model neuro2a in A/J mice (Sigal R.K. *et al.* (1991), *J. Pediatr. Surg.* **26** pp 389-96). On D2 after injection of these tumor cells, the animals (n=6 per group) receive 100 µl of sodium chloride (control group -◆-), or 50 µg of PT1 injected i.p. (-■-) or s.c. at a distance from the tumor (-π-), in 100 µl of sodium chloride.

- Figure 7 illustrates the effect of stabilizing an oligonucleotide (SEQ ID NO: 9 5'-TGACTGTGAACGTTATAGATGA-3') via a linkage of the type phosphorothioate (PT), phosphodiester (PDE), methylphosphonate (MP); phosphodiester stabilized in 3' by a dideoxycytosine base (3') or mixed: phosphodiester with the first 3 linkages in 5' and the last three linkages in 3' of phosphorothioate type (mixed), on the antitumor activity in a subcutaneous glial tumor model. On D2 after injection of the tumor cells, the groups of animals receive, subcutaneously, at the tumor site, sodium chloride (NaCl control, n=9) or 50 µg of the oligonucleotides PT (n=9), PDE (n=8), MP (n=9), 3' (n=7) and mixed (n=9). The volume of the tumor is evaluated on D10. The results are expressed as mean ± s.e.m.

- Figures 8 to 11 illustrate the effect of the sequences 5'-purine-purine-CG-pyrimidine-pyrimidine-X_1X_2-3' on the modulation of the antitumor activity in a subcutaneous glial tumor model. On D2 after injection of the tumor cells, the groups of animals (n= 6) receive, subcutaneously, at the tumor site, sodium chloride (NaCl control) or 50 µg of the oligonucleotides (SEQ ID NO: 2 to 13). The volume of the tumor is evaluated on D8 (Figures 8 to 10) or on D10 (Figure 11). The results are expressed as mean ± s.e.m.:

- Figure 8 illustrates the effect of the oligonucleotide sequences on the antitumor effectiveness.

- Figure 9 illustrates the effect of the sequence of the hexameric motif 5'-purine-purine-CG-pyrimidine-pyrimidine-3' and of the adjacent sequences on the antitumor effectiveness of the oligonucleotides.

- Figure 10 illustrates the effect of the 2 bases (X_1X_2) adjacent to the 3' sequence of the hexameric motif 5'-purine-purine-CG-pyrimidine-pyrimidine-3' on the antitumor effectiveness of the oligonucleotides.

- Figure 11 illustrates the effect of various sequences X_1X_2 on the antitumor effectiveness of the oligonucleotides.

The examples which follow illustrate the invention without, however, limiting it to these particular embodiments.

Example 1: Effect of an intratumoral injection or of an intraperitoneal injection of PT1 (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3') on the survival of the animals, in the glioma model CNS1 in the brain of Lewis rats

1. Procedure:

CNS1 glioma cells cultured *in vitro* are grafted into the brain of healthy Lewis rats, in a proportion of 10^5 cells in the right parietal cortex of the rats (Kruse C. A. *et al.* (1994), *J. Neurooncol.* **22** pp 191-200).

a) *intratumoral injection*:

50 µg of PT1 in 7 µl of sodium chloride are injected at the tumor site, 1, 5 or 9 days after the graft (group treated on D1, n=6; group treated on D5, n=8; group treated on D9, n=4); a control group (n=14) receives sodium chloride.

b) *intraperitoneal injection:*

50 µg of PT1 are injected intraperitoneally on D1 (n = 5); a control group receives sodium chloride (n = 5).

2. Results:

a) *intratumoral injection*:

They are illustrated in figure 1.

The control group shows a mean survival of 15 days and all the animals die before the 23rd day.

The survival of the animals treated with PT1 is greatly increased, with long-term survivals (>90 days) of 67% ($p<0.01$), of 88% ($p<0.002$) and of 50% ($p<0.02$) for the rats treated on D1, D5 and D9, respectively.

All the dead animals exhibit brain tumors at autopsy.

In the surviving rats, none show neurological symptoms and no tumor is found at autopsy carried out on D90.

The histological study of the brains reveals no inflammatory, demyelinating or toxic lesion in the parenchyma adjacent to the injection site.

b) *intraperitoneal injection*:

Under these conditions, the PT1 has no significant effect.

Example 2: Comparison of the effects of an intratumoral injection of PT1 (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3') on the survival of the animals, in the glioma model CNS1 of Lewis rats, with that of an oligodeoxynucleotide (IMM) comprising a nonimmunostimulatory octanucleotide sequence (SEQ ID NO: 1 5'-TGACTGTGAAGGTTAGAGATGA-3')

1. Procedure:

CNS1 glioma cells cultured *in vitro* are grafted into the brain of healthy Lewis rats, in a proportion of 10^5 cells in the right parietal cortex of the rats (Kruse C. A. *et al.* (1994), *J. Neurooncol.* **22** pp 191-200).

On D1 after the graft carried out under the conditions described in example 1, the rats receive an intratumoral injection of 50 µg of IMM dissolved in

7 µl of sodium chloride, or the vehicle alone (n=5 per group).

2. Results:

The lifespan is not statistically different between the control group, having received the sodium chloride, and the treated group, having received the IMM.

Thus, an oligonucleotide which does not contain any immunostimulatory sequence does not make it possible to increase survival, unlike an oligonucleotide which contains such a sequence (Example 1).

Example 3: Effect of an intratumoral injection of PT1 (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3') at various doses on the survival of the animals, in the glioma model CNS1 of Lewis rats

1. Procedure:

On D1 after the graft carried out under the conditions described in Example 1, the rats receive an intratumoral injection of 1 µg, 10 µg or 50 µg of PT1 dissolved in 7 µl of sodium chloride, or the vehicle alone (n=5 per group).

2. Results:

They are illustrated in figure 2.

A survival longer than 90 days is obtained in 60% of the cases ($p<0.01$) after a single injection of 50 µg, and in 20% of the cases (not significant) after a dose of 10 µg.

There is no survivor after a dose of 1 µg (n=5).

All the control rats died.

In the surviving rats, none exhibited neurological symptoms and no tumor is found at autopsy carried out on D90.

Example 4: Investigation of the mechanism of the effects of PT1 (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3'), *in vitro* and *in vivo*, on the CNS1 glioma cells

1. Procedure:

a) *in vitro*

CNS1 glioma cells are placed in culture on D0. On D1, PT1 at concentrations of 0.05 µM, 0.5 µM and of 5 µM is added to these cells and, on D3, the cells are treated with trypsin and their viability is measured.

b) *in vivo*: see procedure of example 1.

2. Results:

a) *in vitro*

PT1, at concentrations of 0.05 µM, 0.5 µM and of 5 µM has no direct cytotoxic action on the CNS1 cells after culturing for 48 hours.

b) *in vivo*

On the other hand, the immunohistochemical studies show that the injection of 50 µg of PT1 in the tumor triggers a massive infiltration of NK cells, of $CD8^+$ T lymphocytes, of macrophages and of microglial cells, whereas the injection of sodium chloride has no effect.

These results suggest that the action of the PT1 is due to activation of the immune system at the tumor site.

Example 5: Effect of an intratumoral injection of PT1, at a tumor site, on the development of a tumor grafted simultaneously, at a distance from this site.

1. Procedure:

The tumor cells are grafted under the conditions described in example 1, at two separate sites 4 mm apart.

On D5 after the graft, a group of rats (n=7) receives an intratumoral injection of 50 µg of PT1 dissolved in 7 µl of sodium chloride at just one of these sites, and the control group (n=6) receives the vehicle alone.

2. Results:

All the rats of the control group die within less than 25 days, whereas 44% of the rats of the group treated with PT1 have a prolonged survival (>90 days), ($p<0.05$).

These results show that the oligonucleotide PT1 has an effect at distance and that the immune response induced at the injection site prevents the development of a tumor grafted simultaneously, at a distance from this site.

Example 6: Study of the immune memory at 3 months in the glioma model CNS1 of Lewis rats, after injection of PT1 (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3')

1. Procedure:

In rats (n=5) which had been treated with 50 µg of PT1 on D5 after the graft, and which had survived due to this treatment with PT1, a new graft of 10^6 cells is carried out 12 weeks later, in another site of the cerebral cortex, under the conditions described in example 1. In parallel, a graft of 10^5 cells is carried out in rats which had not been treated beforehand.

2. Results:

At 90 days, all the animals previously treated with PT1 survived without further treatment.

The histological analysis shows that there is no residual tumor, both for the first site of implantation of the tumor cells and for the second site.

All the control animals died.

These results show that the oligonucleotides have a sustained effect which makes it possible to prevent the development of a tumor, several weeks after the injection of said oligonucleotide. The "memory effect" observed indicates that the oligonucleotide PT1 activates the setting up of a specific antitumor immune response.

Example 7: Effect of an intratumoral injection of PT1 (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3') or of an oligodeoxynucleotide (IMM) comprising a nonimmunostimulatory octanucleotide sequence (SEQ ID NO: 1 5'-TGACTGTGAAGGTTAGAGATGA-3'), in a subcutaneous glial tumor model.

1. Procedure:

CNS1 glioma cells cultured *in vitro* are injected subcutaneously into healthy Lewis rats, in a proportion of $2x10^6$ cells in the right flank (Kruse C. A. *et al.* (1994), *J. Neurooncol.* **22** pp 191-200).

This model makes it possible to monitor more accurately the growth of the tumor, which can be easily evaluated every day in the live animal. In this model, 100% of the animals injected develop a tumor which grows for at least 2 weeks.

Next, on D2 after the injection of the tumor cells, 50 µg or 100 µg of PT1, or 50 µg of IMM, in 100 µl of sodium chloride, are injected into the tumor site (group treated with 50 µg of PT1, n=9; group treated with 100 µg of PT1, n=6; group treated with 50 µg of IMM, n=9); a control group (n=9) receives 100 µl of sodium chloride.

The tumor growth is measured every two days and the tumor volume is estimated using the formula:
Vol=(length x width x width x π)/6.

The animals are sacrificed on D12 after the injection of the tumor cells.

2. Results:

They are illustrated in figure 3.

In the control group, 9 animals out of 9 developed a tumor, with a mean tumor volume on D12 of approximately 900 mm^3.

In the group treated with IMM, 9 animals out of 9 developed a tumor, with a mean tumor volume on D12 of approximately 1 100 mm^3.

In the group treated with 50 µg PT1, 7 animals out of 9 developed a tumor, with a mean tumor volume on D12 of approximately 400 mm^3, whereas in the group treated with 100 µg PT1, only 3 animals out of 6 developed a tumor, with a mean tumor volume on D12 of approximately 200 mm^3.

This set of results confirms, therefore, that the PT1 had a marked antitumor effect, linked to the presence of an immunostimulatory sequence.

This effect is dose dependent.

Example 8: Effect of an intratumoral injection of PT1 (phosphorothioate oligodeoxynucleotide) or of PE1 (non-stabilized oligodeoxynucleotide) in a subcutaneous glial tumor model; PT1 and PE1 both having the same immunostimulatory sequence (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3')

1. Procedure:

CNS1 glioma cells cultured *in vitro* are injected subcutaneously into healthy Lewis rats, in a proportion of $2x10^6$ cells in the right flank, under the conditions described in example 7.

Next, on D2 after the injection of the tumor cells, 100 µg of PT1 or 100 µg of PE1 are injected into the tumor site (group treated with 100 µg of PT1 in 100 µl of sodium chloride, n=6; group treated with 100 µg of PE1 in 100 µl of sodium chloride, n=6); a control group (n=6) receives 100 µl of sodium chloride.

The tumor growth is measured every two days and the tumor volume is measured as described in Example 7.

The animals are sacrificed on D12 after the injection of the tumor cells.

2. Results:

They are illustrated in figure 4.

In the control group, 6 animals out of 6 developed a tumor, with a mean tumor volume on D12 of approximately 1 200 mm^3.

In the group treated with PE1, 6 animals out of 6 developed a tumor, with a mean tumor volume on D12 of approximately 1 000 mm^3.

In the group treated with 100 µg PT1, only 3 animals out of 6 developed a tumor, with a mean tumor volume on D12 of approximately 200 mm^3.

Example 9: Effect of an intratumoral injection of PT1 (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3') and of IMM (SEQ ID NO: 1 5'-TGACTGTGAAGGTTAGAGATGA-3') in the neuroblastoma model neuro2a in A/J mice

1. Procedure:

The tumor is obtained by injecting one million neuro2a cells into the right flank of A/J mice (Sigal R. K. *et al.* (1991), *J. Pediatr. Surg.*, **26** pp 389-96). This tumor grows in 15-20 days, generally resulting in the death of the animal or making it necessary to sacrifice it.

On D2 after the injection of these tumor cells, 50 µg or 100 µg of PT1, or 50 µg of IMM, in 100 µl of sodium chloride, or 100 µl of sodium chloride (control group), are injected into the same site (n=6 animals per group).

The tumor growth is measured every four days and the tumor volume is measured as indicated in example 7.

The animals are sacrificed on D22 after the injection of these tumor cells.

2. Results:

They are illustrated in figure 5.

In this model, the mean tumor volume on D22 is approximately 800 mm^3 in the control group, approximately 1200 mm^3 in the group treated with 50 µg of IMM, and approximately 200 mm^3 in the groups treated with 50 µg or 100 µg of PT1.

Example 10: Effect of a subcutaneous or intraperitoneal injection of PT1 (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3') at the dose of 50 µg, in the neuroblastoma model neuro2a in A/J mice

1. Procedure:

The tumor is obtained according to the procedure described in example 9.

On D2 after the injection of the tumor cells, 50 µg of PT1 in 100 µl of sodium chloride, or 100 µl of sodium chloride (control group), are injected either subcutaneously at a distance from the tumor, or intraperitoneally (n=6 animals per group).

The tumor growth is measured every four days and the tumor volume is measured as indicated in example 7.

The animals are sacrificed on D22 after the injection of the tumor cells.

2. Results:

They are illustrated in figure 6.

In this model, the mean tumor volume on D22 is approximately 1 000 mm^3 in the control group, approximately 400 mm^3 in the group treated with 50 µg of PT1 injected subcutaneously and approximately 500 μm^3 in the group treated with 50 µg of PT1 injected intraperitoneally.

Example 11: Effect of repeated subcutaneous injection of PT1 (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3') or of IMM (SEQ ID NO: 1 5'-TGACTGTGAAGGTTAGAGATGA-3') at the dose of 10 µg, for 15 days, in the neuroblastoma model neuro2a in A/J mice

1. Procedure:

The tumor is obtained according to the procedure described in Example 9.

The tumor growth is measured regularly in all the animals, and when the diameter of the tumor reaches 5 mm, PT1 is injected subcutaneously, around the tumor, for 15 days, at the dose of 10 µg per day in 100 µl of sodium chloride (group treated with PT1, n=7) or IMM is

injected subcutaneously, around the tumor, for 15 days, at the dose of 10 µg per day in 100 µl of sodium chloride (group treated with IMM, n=4) or 100 µl of sodium chloride is injected subcutaneously, around the tumor, for 15 days (control group, n=6).

2. Results:

In the control group and in the group treated with the IMM, the tumor growth is not slowed down and all the animals of these two groups die from their tumor.

In the group treated with PT1, complete disappearance of the tumor, with no long term recurrence, is observed in 3 mice; in 3 others, the tumors are stabilized for 3 weeks but then recommence their progression until the animals die.

These results show that the stabilized immunostimulatory oligonucleotides used according to the invention have a marked intrinsic antitumor effect, linked to the presence of the immunostimulatory sequence and to their stabilization.

Example 12: Effect of stabilizing an oligonucleotide (SEQ ID NO: 9 5'-TGACTGTGAACGTTATAGATGA-3') on the antitumor activity, in a subcutaneous glial tumor model

1. Procedure:

CNS1 glioma cells cultured *in vitro* are injected subcutaneously into healthy Lewis rats, in a proportion of $2x10^6$ cells in the right flank (Kruse C. A. *et al.* (1994), *J. Neurooncol.* **22** pp 191-200).

On D2 after the injection of the tumor cells, 50 µg of the oligonucleotides having the various chemical linkages are injected into the tumor site and the tumor volume is measured on D10 (groups treated with an oligonucleotide of linkage: phosphorothioate (PT, n=9), phosphodiester (PDE, n=8), methylphosphonate (MP, n=9), phosphodiester stabilized in 3' by a dideoxycytosine base (group 3', n=7), or mixed:

phosphodiester with the first three linkages in 5' and the last three linkages in 3' of the phosphorothioate type (mixed group, n=9). The control group receives 100 µl of sodium chloride (NaCl n=9).

2. Results:

They are illustrated in figure 7.

In this model, the most effective ODNs are the oligonucleotides of type phosphorothioate, stabilized in 3', or mixed, with a decrease in the tumor volume of 50%, 53% and 34%, respectively, with respect to the volume of the controls.

Example 13: Effect of the sequences 5'-purine-purine-CG-pyrimidine-pyrimidine-X_1X_2-3' on the modulation of the antitumor activity

1. Procedure:

CNS1 glioma cells cultured *in vitro* are injected subcutaneously into healthy Lewis rats, in a proportion of $2x10^6$ cells in the right flank (Kruse C. A. *et al.* (1994), *J. Neurooncol.* **22** pp 191-200).

On D2 after the injection of the tumor cells, 50 µg of the various oligonucleotides (SEQ ID NO: 2 to 13) are injected into the tumor site and the tumor volume is measured on D10 (figures 8 to 10) or on D8 (figure 11).

2. Results:

2.1. Effect of the oligonucleotide sequence on the antitumor effectiveness

The results are illustrated in figure 8.

The oligonucleotide PT1 (SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3') used above (examples 1 to 10) is less effective than the oligonucleotide An 2 (SEQ ID NO: 8 5'-TGCCAGTGACGTCATGTGAC-3').

The difference in effectiveness of these two oligonucleotides is linked either to the sequence of the hexameric motif 5'-purine-purine-CG-pyrimidine-pyrimidine-3' comprising the nonmethylated CG motif

(underlined sequence), or to the sequences adjacent to this motif.

2.2. Effect of the sequence of the hexameric motif 5'-purine-purine-CG-pyrimidine-pyrimidine-3' and of the adjacent sequences on the antitumor effectiveness of the oligonucleotides.

The results are illustrated in figure 9.

They show that oligonucleotides having a different hexameric motif, GACGTC (An2, SEQ ID NO: 8 5'-TGCCAGTGACGTCATGTGAC-3') or AACGTT (An21, SEQ ID NO: 10 5'-TGCCAGTAACGTTATGTGAC-3'), and identical adjacent sequences have the same antitumor effectiveness.

Consequently, the differences in effectiveness observed, in example 2.1, between the oligonucleotides PT1 and An2 are linked to the nature of the sequences adjacent to the hexameric motif. The optimum antitumor sequences are found in the adjacent sequences of the oligonucleotide An2.

2.3. Effect of the 2 bases (X_1X_2) adjacent to the 3' sequence of the hexameric motif 5'-purine-purine-CG-pyrimidine-pyrimidine-3' on the antitumor effectiveness

The results are illustrated in figure 10.

They show that the 2 bases adjacent to the 3' sequence of the hexameric motif modulate, all by themselves, the effectiveness of the oligonucleotides, since 2 oligonucleotides which are identical along their entire sequence with the exception of these 2 nucleotides have different effectivenesses, of the order of those previously observed with the oligonucleotides of example 2.1. Thus, the oligonucleotide An 14 (SEQ ID NO: 3 5'-TGACTGTGAACGTTCCAGATGA-3') is less effective than the oligonucleotide An 15 (SEQ ID NO: 9, 5'-TGACTGTGAACGTTATAGATGA-3'). The nucleotides AT positioned 3' of the hexameric motif (An2 (figure 8) and An 15 (figure 10)) make it possible to increase the antitumor effectiveness, whereas the nucleotides CC

(An 14, figure 10) and CG (PT1, figure 8) have less marked antitumor effects.

2.4. Effect of various sequences X_1X_2 on the antitumor effectiveness

The results are illustrated in figure 11.

The optimum antitumor effect is observed with the sequences X_1X_2= AT, AA, CT or TT (An2 SEQ ID NO: 8 5'-TGCCAGTAACGTTAAGTGAC-3'; An22 SEQ ID NO: 11 5'-TGCCAGTAACGTTAAGTGAC-3'; An25 SEQ ID NO: 12 5'-TGCCAGTAACGTCTGTGAC-3'; An27 SEQ ID NO: 13 5'-TGCCAGTAACGTTTTGTGAC-3').

The sequences X_1X_2 = AC, AG, GT and CC and CG (An23 SEQ ID NO: 4 5'-TGCCAGTAACGTTACGTGAC-3'; An24 SEQ ID NO: 5 5'-TGCCAGTAACGTTAGGTGAC-3'; An26 SEQ ID NO: 6 5'-TGCCAGTAACGTTGTGTGAC-3'; An 28 SEQ ID NO: 7 5'-TGCCAGTAACGTTCCGTGAC-3' and PT1 SEQ ID NO: 2 5'-TGACTGTGAACGTTCGAGATGA-3' (see figure 8)) do not improve the antitumor activity of the oligonucleotides having a hexameric motif 5'-purine-purine-CG-pyrimidine-pyrimidine-3'.

These results show that the set of stabilized oligonucleotides of the type 5'-purine-purine-CG-pyrimidine-pyrimidine-X_1X_2-3' with X_1X_2 = AA, AT, CT or TT has optimized antitumor activity.

CLAIMS

1. The use of stabilized oligonucleotides which comprise at least one octameric motif of the type 5'-purine-purine-CG-pyrimidine-pyrimidine-X_1X_2-3', in which the pair X_1X_2 is AT, AA, CT or TT, for preparing a medicament with antitumor activity.

2. The use of stabilized oligonucleotides as claimed in claim 1, characterized in that said oligonucleotides comprise at least one octameric motif selected from the group consisting of: AACGTT-X_1X_2, GACGTT-X_1X_2, AGCGTT-X_1X_2, GGCGTT-X_1X_2, AACGTC-X_1X_2, GACGTC-X_1X_2, AGCGTC-X_1X_2 and GGCGTC-X_1X_2 in which X_1X_2 is AT, AA, CT or TT.

3. The use as claimed in either of claims 1 and 2, characterized in that the oligonucleotide is chosen from the group consisting of the sequences SEQ ID NO: 8 to SEQ ID NO: 48.

4. The use as claimed in any one of claims 1 to 3, characterized in that at least one cytosine of the octameric motif is replaced with a modified cytosine.

5. The use of stabilized oligonucleotides as claimed in any one of claims 1 to 4, characterized in that the stabilized oligonucleotides are selected from the group consisting of phosphorothioates, phosphorodithioates, phosphodiester-phosphorothioate mixed oligonucleotides, methylphosphonates and the oligonucleotides of which at least one end has been stabilized.

6. The use of stabilized oligonucleotides as claimed in any one of claims 1 to 5, characterized in that the oligonucleotides are in single-stranded or double-stranded form.

7. The use of stabilized oligonucleotides as claimed in any one of claims 1 to 6, characterized in that the oligonucleotides comprise at least 8 nucleotides, and preferably between 20 nucleotides and 100 nucleotides.

8. The use of stabilized oligonucleotides as claimed in any one of claims 1 to 7, characterized in that the oligonucleotides are coupled, via covalent, ionic or weak attachments, to a molecule capable of increasing tumor affinity.

9. The use of stabilized oligonucleotides as claimed in any one of claims 1 to 8, for preparing a medicament intended for the treatment of cancers in humans, whatever their nature and their degree of anaplasia.

10. The use of stabilized oligonucleotides as claimed in claim 9, characterized in that the cancer is chosen from the group consisting of cancers of the central and peripheral nervous systems.

11. The use of stabilized oligonucleotides as claimed in claim 9, characterized in that the cancer is chosen from the group consisting of astrocytomas, glioblastomas, medulloblastomas, neuroblastomas, melanomas and carcinomas.

12. The use of stabilized oligonucleotides as claimed in any one of claims 9 to 11, characterized in that the medicine is administered via the intratumoral route.

13. The use of stabilized oligonucleotides as claimed in claim 12, characterized in that the medicine is administered so as to have a dose of 10 to 1 000 µg/g of tumor, at least in a part of the tumor mass.

14. The use of stabilized oligonucleotides as claimed in any one of claims 9 to 11, characterized in that the medicine is administered via a route chosen from the group consisting of the intravenous, intraperitoneal, topical, transdermal, subcutaneous, intra-arterial, pulmonary, nasopharyngeal or oral routes.

15. The use as claimed in any one of claims 9 to 14, characterized in that the stabilized oligonucleotides are combined with any physical or

chemical means which facilitates the production of an effective dose at the tumor site.

16. The use as claimed in any one of claims 9 to 15, characterized in that the oligonucleotides are administered in any pharmaceutically acceptable form.

17. The use as claimed in any one of claims 9 to 16, characterized in that the oligonucleotides are combined with cells of the immune system, adjuvants of immunity, cytokines, antitumor antibodies, tumor extracts, tumor antigens, or irradiated, genetically modified, or normal, tumor cells.

(12) DEMANDE INTERNATIONALE PUBLIÉE EN VERTU DU TRAITÉ DE COOPÉRATION EN MATIÈRE DE BREVETS (PCT)

(19) Organisation Mondiale de la Propriété Intellectuelle
Bureau international

(43) Date de la publication internationale
28 septembre 2000 (28.09.2000)

PCT

(10) Numéro de publication internationale
WO 00/56342 A3

(51) Classification internationale des brevets[7] :
A61K 31/70, C07H 21/00

(21) Numéro de la demande internationale :
PCT/FR00/00676

(22) Date de dépôt international : 17 mars 2000 (17.03.2000)

(25) Langue de dépôt : français

(26) Langue de publication : français

(30) Données relatives à la priorité :
99/03433 19 mars 1999 (19.03.1999) FR

(71) Déposants *(pour tous les États désignés sauf US)* : **ASSISTANCE PUBLIQUE-HOPITAUX DE PARIS** [FR/FR]; 3, avenue Victoria, F-75004 Paris (FR). **INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE** [FR/FR]; 101, rue de Tolbiac, F-75654 Paris Cedex 13 (FR).

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(81) États désignés *(national)* : AU, CA, JP, US.

(84) États désignés *(régional)* : brevet européen (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Publiée :
— *avec rapport de recherche internationale*

(88) Date de publication du rapport de recherche internationale: 26 juillet 2001

En ce qui concerne les codes à deux lettres et autres abréviations, se référer aux "Notes explicatives relatives aux codes et abréviations" figurant au début de chaque numéro ordinaire de la Gazette du PCT.

WO 00/56342 A3

(54) Title: USE OF STABILISED OLIGONUCLEOTIDES FOR PREPARING A MEDICINE WITH ANTITUMOUR ACTIVITY

(54) Titre : UTILISATION D'OLIGONUCLEOTIDES STABILISES POUR LA PREPARATION D'UN MEDICAMENT A ACTION ANTITUMORALE

(57) Abstract: The invention concerns the use of stabilised oligonucleotides comprising at least an octamer motif of the type: 5'-purine-purine-CG-pyrimidine-pyrimidine-X_1X_2-3' wherein the pair X_1-X_2 is AT, AA, CT or TT, for preparing a medicine with antitumour activity.

(57) Abrégé : Utilisation d'oligonucléotides stabilisés qui comprennent au moins un motif octamérique du type: 5'-purine-purine-CG-pyrimidine-pyrimidine-X_1X_2-3', dans lequel la paire X_1-X_2 est AT, AA, CT ou TT, pour la préparation d'un médicament à action antitumorale.

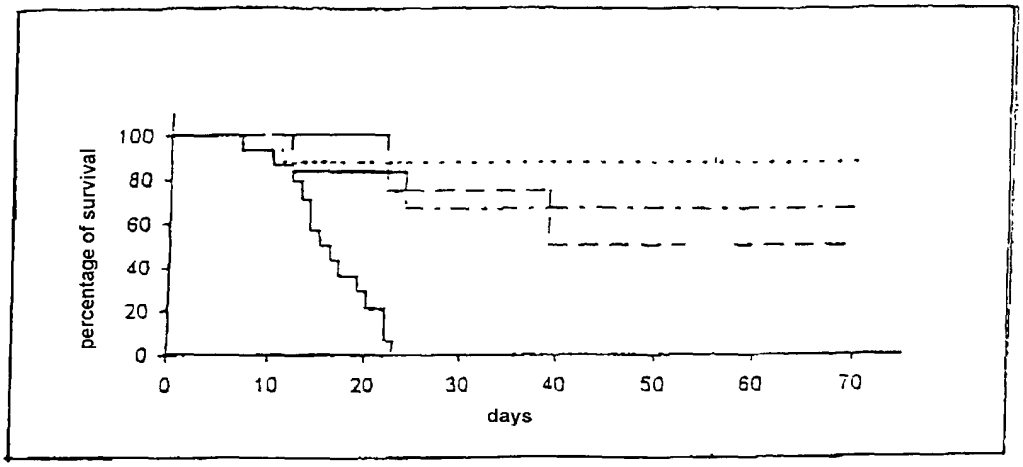
percentage of survival
100
80
60
40
20
0
0
10
20
30
40
50
60
70
days

FIGURE 1

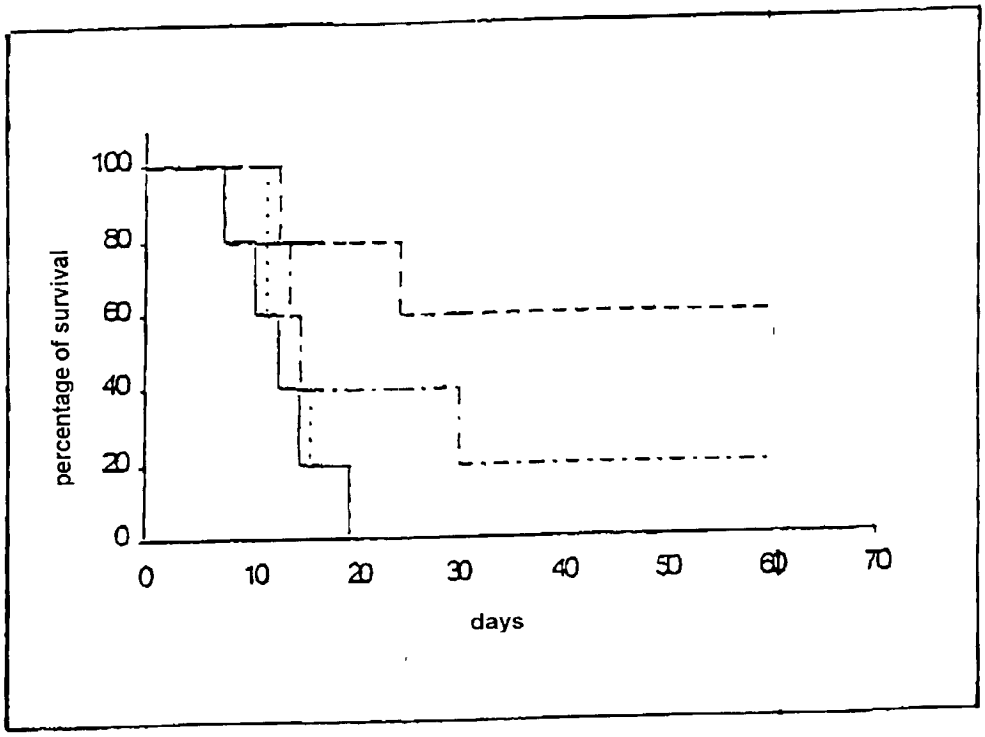
100
80
60
40
20
0
percentage of survival
0
10
20
30
40
50
60
70
days

FIGURE 2

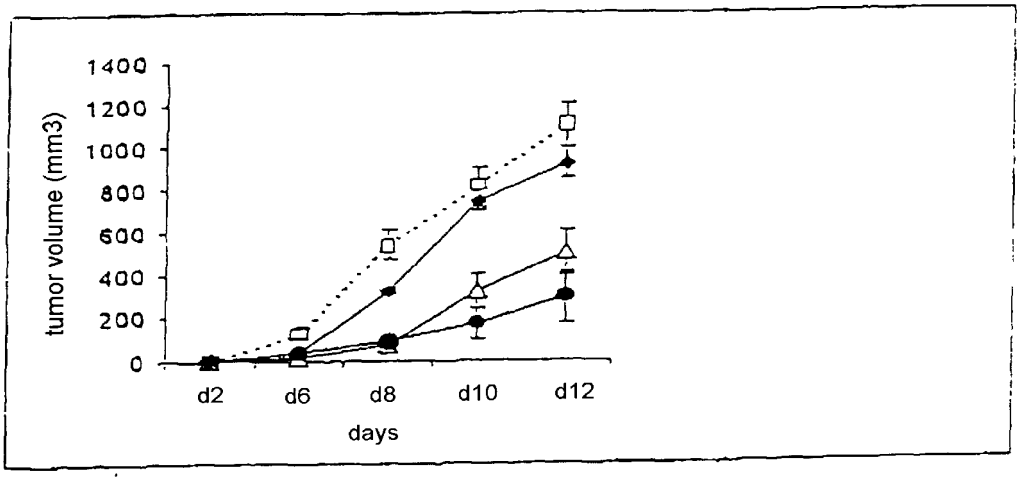
1400
1200
1000
800
600
400
200
0
tumor volume (mm3)
d2
d6
d8
d10
d12
days

FIGURE 3

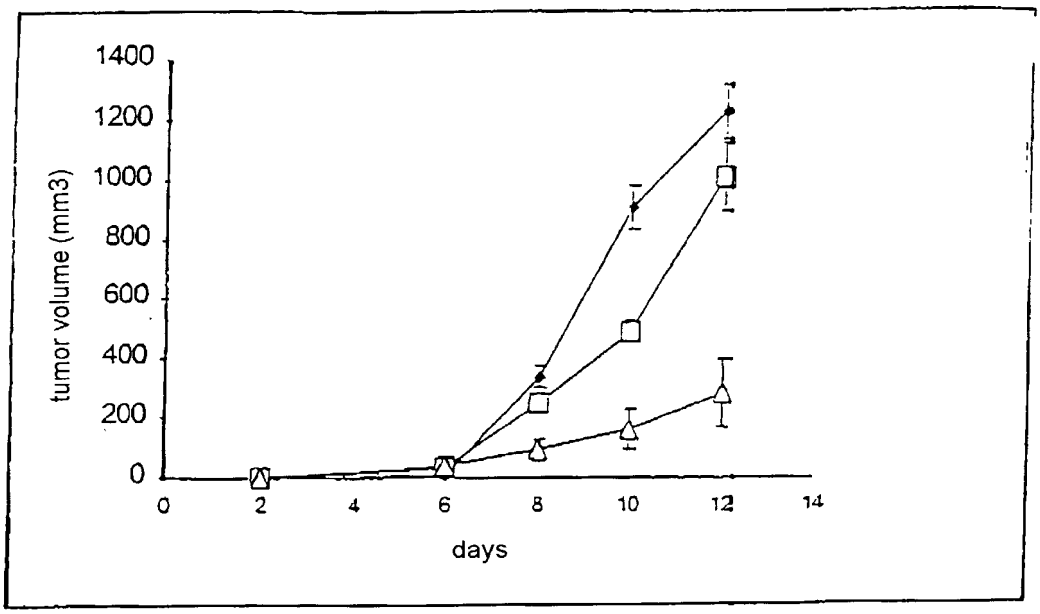
1400
1200
1000
800
600
400
200
0
tumor volume (mm3)
0
2
4
6
8
10
12
14
days

Figure 4

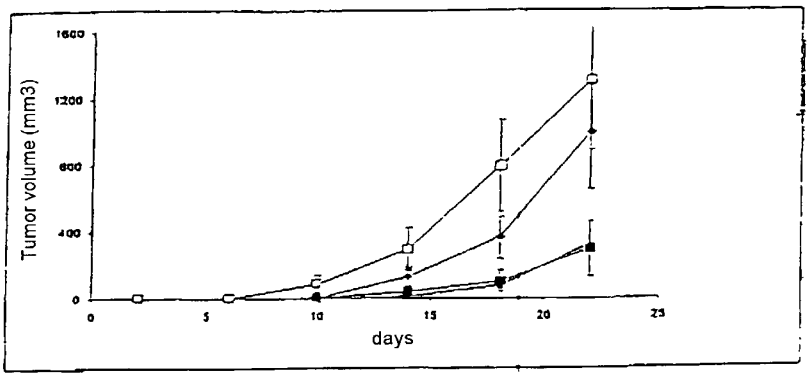

FIGURE 5

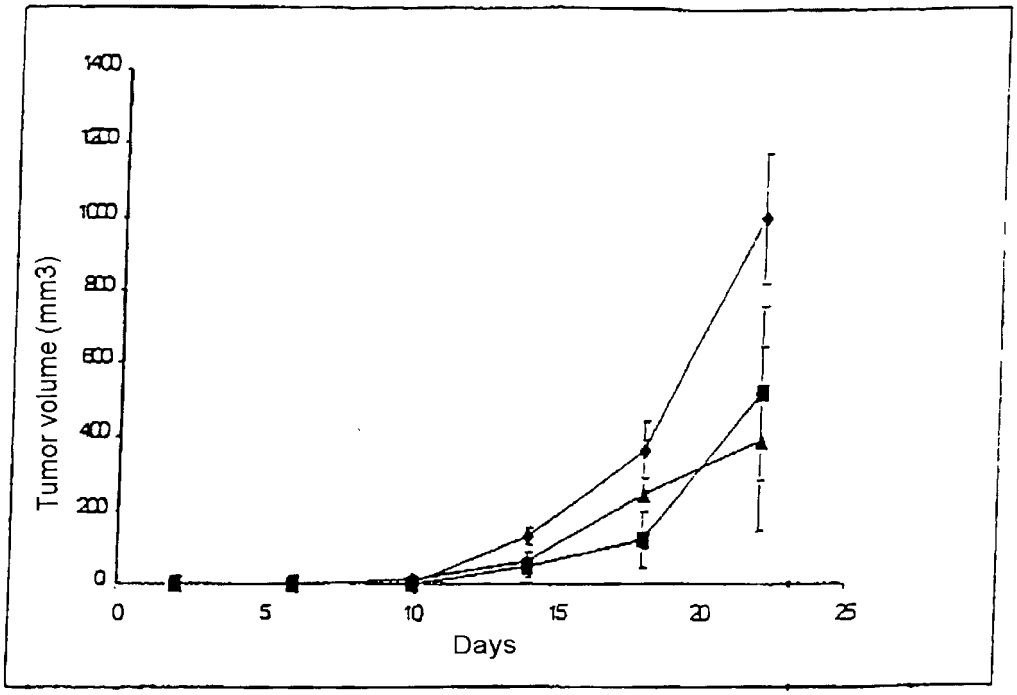
Tumor volume (mm3)
0
200
400
600
800
1000
1200
1400
0
5
10
15
20
25
Days

FIGURE 6

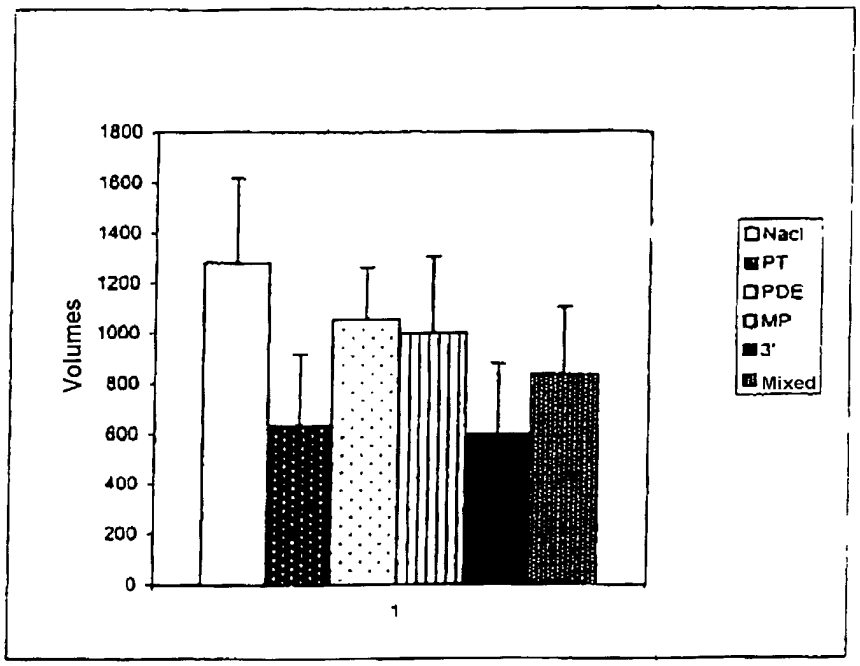
1800
1600
1400
1200
1000
800
600
400
200
0
Volumes
1
Nacl
PT
PDE
MP
3'
Mixed

FIGURE 7

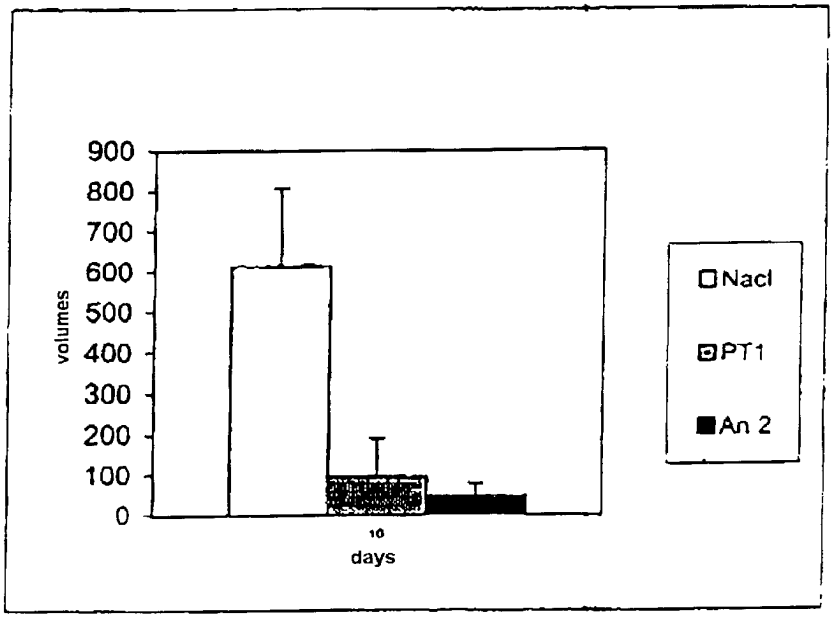
900
800
700
600
500
400
300
200
100
0
volumes
10
days
Nacl
PT1
An 2

FIGURE 8

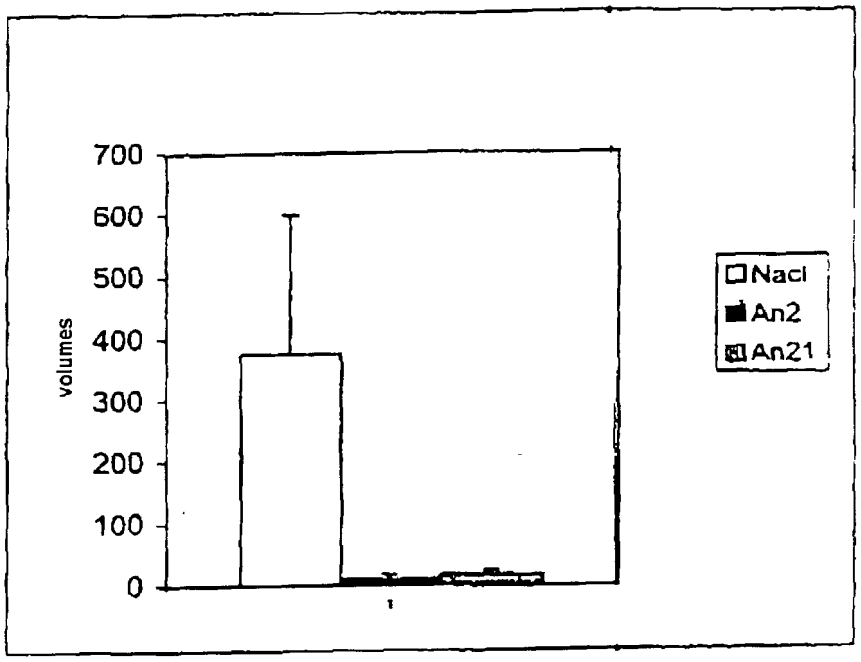

FIGURE 9

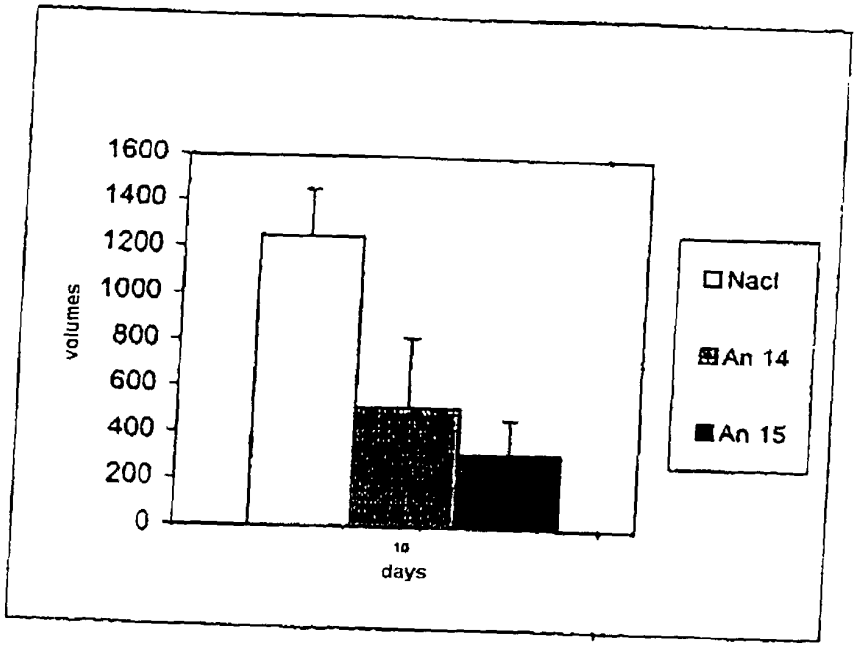
1600
1400
1200
1000
800
600
400
200
0
volumes
10
days
Nacl
An 14
An 15

FIGURE 10

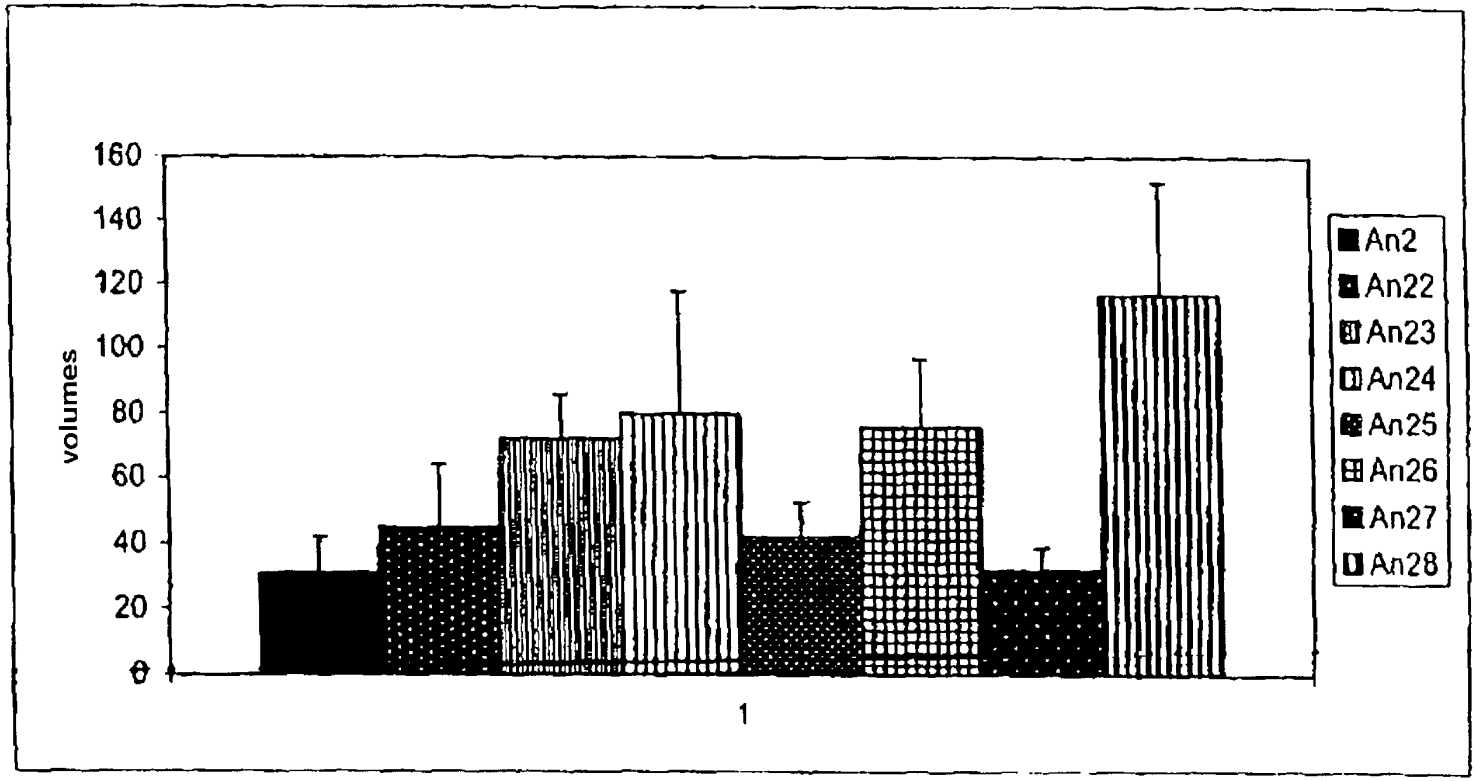

FIGURE 11

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Docket Information
260/264

#4

DECLARATION
Utility Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **USE OF STABILISED OLIGONUCLEOTIDES FOR PREPARING A MEDICAMENT WITH ANTITUMOR ACTIVITY** the specification of which

(Check One) ☐ is attached hereto OR
☒ was filed on September 19, 2001 as United States Application Serial No. 09/______ or PCT International Application No. **09/937,057** and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Date of Filing	Priority Claimed	
			Yes	No
PCT/FR00/00676	FRANCE	MARCH 19, 1999	X	

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date	Status-Patented, Pending or Abandoned

AL

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Docket Information
260/264

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1-00

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INVENTOR'S SIGNATURE _____ DATE 12/20/01

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	POST OFFICE ADDRESS		City	State or Country	Zip Code

INVENTOR'S SIGNATURE _____ DATE _____